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New Perspectives for Cancer Hazard Evaluation by the Report on Carcinogens: A Case Study Using Read-Across Methods in the Evaluation of Haloacetic Acids Found as Water Disinfection By-Products

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Background: Due to the large number of chemicals not yet tested for carcinogenicity but to which people are exposed, the limited number of human and animal cancer studies conducted each year, and the frequent need for a timely response, mechanistic data are playing an increasingly important role in carcinogen hazard identification.

Objectives: To provide a targeted approach to identify relevant mechanistic data in our cancer evaluation of haloacetic acids (HAAs), we used several approaches including systematic review, the 10 key characteristics of carcinogens (KCs), and read-across methods. Our objective in this commentary is to discuss the strengths, limitations, and challenges of these approaches in a cancer hazard assessment.

Methods: A cancer hazard assessment for 13 HAAs found as water disinfection by-products was conducted. Literature searches for mechanistic studies focused on the KCs and individual HAAs. Studies were screened for relevance and categorized by KCs and other relevant data, including chemical properties, toxicokinetics, and biological effects other than KCs. Mechanistic data were organized using the KCs, and strength of evidence was evaluated; this information informed potential modes of action (MOAs) and read-across-like approaches. Three read-across options were considered: evaluating HAAs as a class, as subclass(es), or as individual HAAs (analog approach).

Discussion: Because of data limitations and uncertainties, listing as a class or subclass(es) was ruled out, and an analog approach was used. Two brominated HAAs were identified as target (untested) chemicals based on their metabolism and similarity to source (tested) chemicals. In addition, four HAAs with animal cancer data had sufficient evidence for potential listing in the Report on Carcinogens (RoC). This is the first time that the KCs and other relevant data, in combination with read-across principles, were used to support a recommendation to list chemicals in the RoC that did not have animal cancer data. https://doi.org/10.1289/EHP5672

Introduction

Cancer hazard evaluation and risk assessment are expected to rely increasingly on the use of mechanistic data and predictive tools due to increasing costs of testing individual chemicals, the need for reduction in experimental animal usage, the paucity of toxicity data on most chemicals used in commerce, and the need for a timely response (Guyton et al. 2009). Thus, there is a critical need for cost- and time-efficient approaches that enable the use of mechanistic information in chemical hazard assessments. In response to this need, alternative testing methods (e.g., molecular epidemiology, high-throughput assays, toxicogenomics), along with computational tools [e.g., read-across, *in vitro* to *in vivo* extrapolation, quantitative structure–activity relationship models (QSARs)], are active areas of research and development (Patlewicz et al. 2013; Lan et al. 2016; Chiu et al. 2018; Honda et al. 2019). However, this increasing reliance on mechanistic data and alternative methods poses challenges, as standard practices for conducting systematic reviews of mechanistic data have not been fully developed.

We used systematic review methods, including use of the 10 key characteristics of carcinogens (KCs) for evaluating mechanistic data (Smith et al. 2016), in the cancer hazard assessment of 13 haloacetic acids (HAAs) found as water disinfection by-products for potential listing in the Report on Carcinogens (RoC) (NTP 2018b). In our approach, mechanistic data for each of the 13 HAAs were organized by KCs and reviewed for the strength of evidence supporting each KC. The KCs (e.g., genotoxicity, electrophilicity) were identified based on a review of known human carcinogens (Group 1) listed by the International Agency for Research on Cancer (IARC) and are a means to identify relevant mechanistic literature without presupposition of a mode of action (Smith et al. 2016; Guyton et al. 2018).

We also incorporated read-across approaches in the RoC cancer hazard assessment process for HAAs. Read-across is an emerging alternative approach for filling data gaps for an untested (target) chemical(s) based on a direct comparison to a similar tested (source) chemical(s). The approach can be based on analog (e.g., one source chemical to one target chemical) or chemical category (e.g., many to one or many to many) approaches (Patlewicz et al. 2015; Schultz et al. 2015). In addition to assessing structural similarity between the source and target chemicals, it is also important to assess the degree of similarity for other factors [e.g., reactivity, metabolism, physicochemical properties, and mechanism(s) of action] as well as the primary sources of uncertainty (Wu 2010). In cases where similarity in properties between the source chemical dataset and target chemical(s) can be established, the level of uncertainty may be reduced such that the hazard assessment is considered comparable to having direct data on the target chemical(s) (Wu 2010; Blackburn and Stuard 2014).

The process for listing a substance in the RoC is based on published RoC listing criteria that rely on human and animal data evaluations and mechanistic data (NTP 2018a). In cases where human and/or animal carcinogenicity data are sufficient to meet listing criteria, mechanistic data are not required to list a substance as a cancer hazard. However, the RoC criteria also include listing options based on mechanistic evidence alone (i.e., "the agent, substance, or mixture belongs to a well-defined, structurally related class of substances whose members are listed in a previous RoC, or there is convincing relevant

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information that the agent acts through mechanisms indicating it would likely cause cancer in humans"). The National Toxicology Program (NTP) recommends substances for listing in the RoC, but the final decision to add the listing or make any other change is made by the secretary of the Department of Health and Human Services.

In this commentary, we present an overview of the recent evaluation of HAAs as a test case for applying the principals of read-across and using mechanistic data to support a listing recommendation in the RoC (NTP 2018b). HAAs are derivatives of acetic acid with one or more halogen atoms (e.g., chlorine, bromine, or iodine) attached to the alpha carbon atom (Figure 1). HAAs are formed during water treatment when chlorine-based disinfectants react with organic material naturally present in the water source. Over 600 water disinfection by-products have been identified, but few have been tested for potential health effects—an example of the need to establish alternative methods for health evaluation of chemicals (Richardson et al. 2007). HAAs, along with trihalomethanes (i.e., chloroform, bromoform, bromodichloromethane, and chlorodibromomethane), are the two most prevalent classes of disinfection by-products in drinking water. While this commentary provides some discussion of the mono- and iodinated HAAs that were included in the cancer evaluation, the focus is on the di- and tri-HAAs containing chlorine and/or bromine. No fluorinated HAAs were included because they have not been identified as water disinfection by-products.

As to the RoC monograph on HAAs, the primary objective of this commentary is to discuss the approach and methods we used to evaluate the carcinogenic hazards of HAAs, review the primary strengths and limitations of the data and approach, and discuss lessons learned from our experience and recommendations for future directions for the NTP and others making cancer hazard assessments. Although some of the key findings and conclusions from the monograph are mentioned, the full details of the

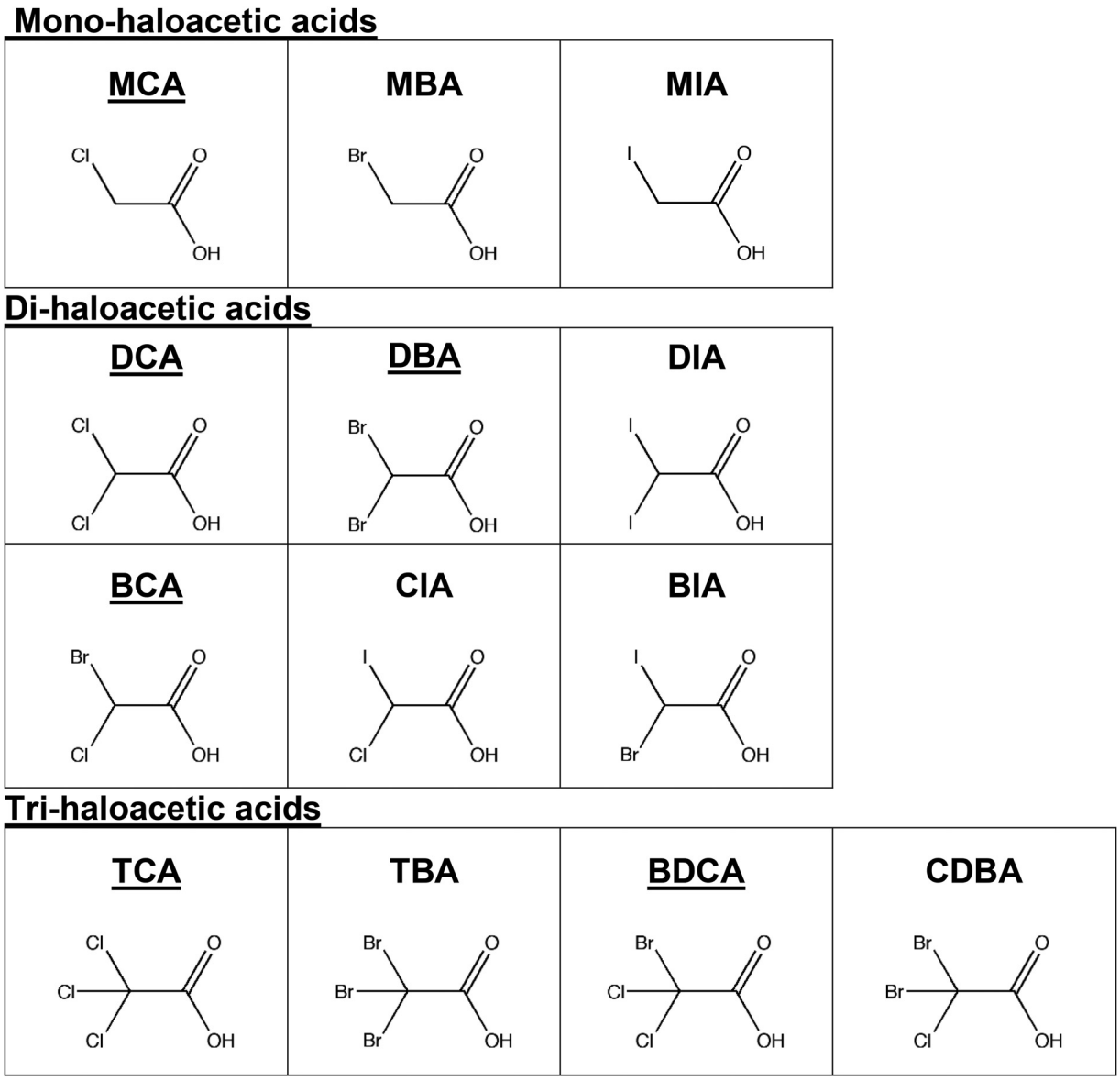

Figure 1. Chemical structures for 13 haloacetic acids found as water disinfection by-products. Underlined chemicals are those that have animal cancer data (note that only DCA, DBA, BCA, and BDCA have sufficient animal cancer data to meet the Report on Carcinogens listing criteria). Note: BCA, bromochloroacetic acid; BDCA, bromodichloroacetic acid; BIA, bromoiodoacetic acid; CDBA, chlorodibromoacetic acid; CIA, chloroiodoacetic acid; DBA, dibromoacetic acid; DCA, dichloroacetic acid; DIA, diiodoacetic acid; MBA, monobromoacetic acid; MCA, monochloroacetic acid; MIA, monoiodoacetic acid; TBA, tribromoacetic acid; TCA, trichloroacetic acid.

evaluation and data supporting the conclusions are provided in the final "Report on Carcinogens Monograph on Haloacetic Acids Found as Water Disinfection By-Products" (NTP 2018b). Key elements of our approach included systematic review, KCs, and read-across methods.

Methods

The approach for preparing the "Report on Carcinogens Monograph on Haloacetic Acids Found as Water Disinfection By-Products" is illustrated in Figure 2 and began with *a*) scoping and problem formulation activities, such as systematic literature searches and evidence mapping, including the 10 KCs and other relevant data (see Steps A and B in Figure 2), leading to protocol development (Step C) followed by *b*) preparation of the cancer hazard evaluation (Step D), and *c*) peer review of the draft monograph by a panel of experts (Step E). As per the RoC review, a listing of a substance in the RoC requires review and approval by the secretary of the Department of Health and Human Services (NTP 2018a). These steps are discussed in detail in the following paragraphs.

Scoping and Problem Formulation Activities

Systematic literature review. References identified in the literature search were uploaded to the publicly available Health Assessment Workspace Collaborative (HAWC) management system and screened for relevance (HAWC 2019). In addition to the primary search of published literature in three citation databases (NCBI 2019; Scopus 2019; Web of Science 2019) and authoritative reviews, several toxicological databases [e.g., Tox21 (NTP 2018c), ToxCast (U.S. EPA 2019b), and NTP's Chemical Effects in Biological Systems (NTP 2019a)] were also searched for relevant data.

Evidence mapping. References that met the inclusion criteria were organized according to relevant topics, including human exposure, toxicokinetics (i.e., the processes and rates of absorption, distribution, metabolism, and excretion), animal cancer data, human cancer studies, and mechanistic data. The body of epidemiological data was considered inadequate to evaluate the relationship between human cancer and exposure to HAAs, as only one study on exposure to a specific HAA was identified. The mechanistic literature was organized according to the KCs (Figure 2). Based

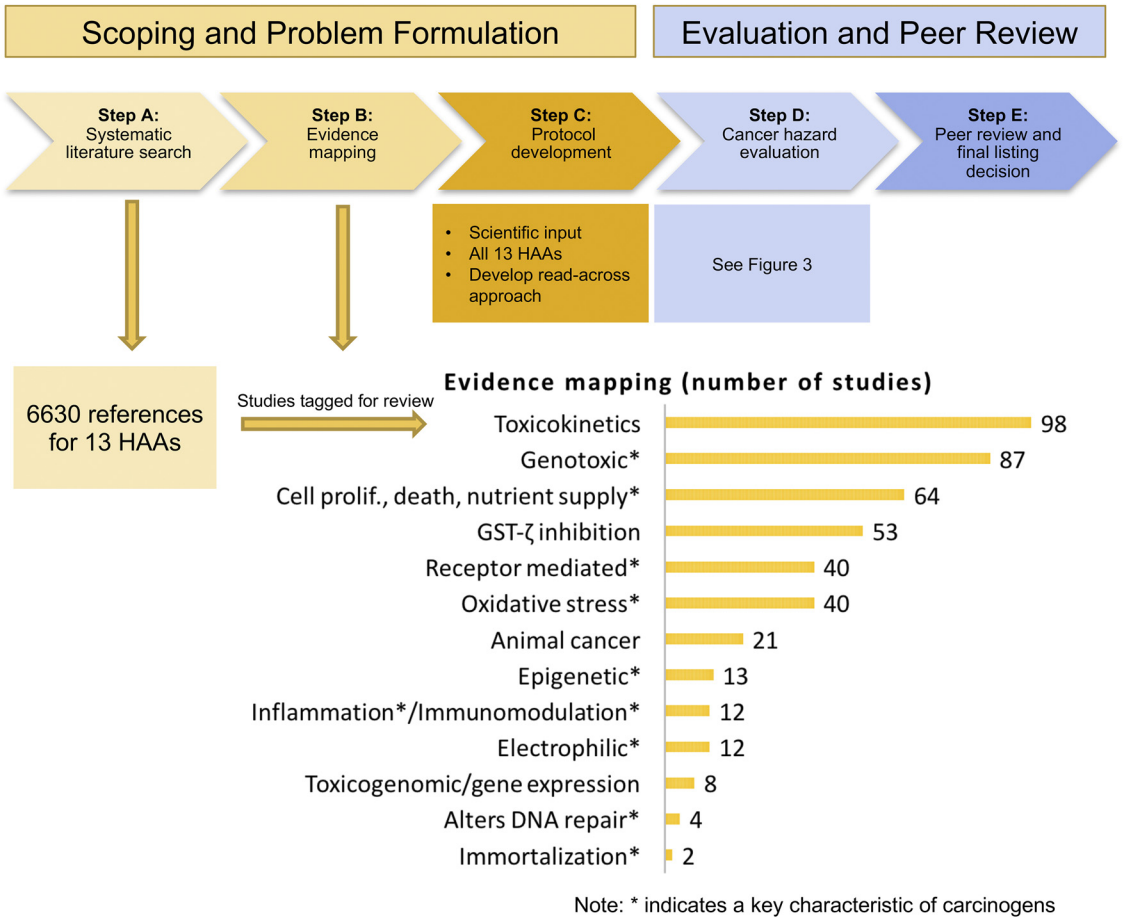

Figure 2. Report on Carcinogens (RoC) review process for haloacetic acids (HAAs). The review process began with "Scoping and Problem Formulation," which consisted of three steps. Step A included systematic literature searches of three scientific citation databases (NCBI 2019; Scopus 2019; Web of Science 2019), which identified more than 6,600 references for the 13 HAAs under review. These studies were screened and tagged in Health Assessment Workspace Collaborative (HAWC) for relevant human, animal, and mechanistic data. In Step B, the mechanistic and other relevant studies were tagged and mapped according to toxicokinetic data and the 10 key characteristics of carcinogens, and animal cancer studies as shown in the evidence mapping graph insert above (Note: the 10 KCs are indicated by asterisks in the graph). Evidence mapping provides an overview of the available data and is a part of methods development prior to cancer hazard assessments. In Step C, the information obtained in Steps A and B was used to develop the RoC protocol for HAAs. The first three steps were followed by two additional steps for "Evaluation and Peer Review." In Step D, the cancer hazard evaluation process was conducted as directed by the RoC protocol. In Step E, the RoC monograph, which included application of potential read-across approaches, was peer-reviewed, and an RoC listing recommendation was made. Note: DNA, deoxyribonucleic acid; GST-ζ, glutathione S transferase zeta.

on the availability of animal carcinogenicity data for six HAAs, clear trends in chemical properties, and comparative toxicokinetic and mechanistic studies (KCs or other relevant biological effects) for all 13 HAAs, this was considered to be a good test case to apply mechanistic and read-across methodology in the RoC evaluation.

Protocol development. The protocol (NTP 2017) applies general methods for the RoC cancer hazard assessments [available in the *Handbook for Preparing Report on Carcinogens Monographs* (NTP 2015a)] to special issues identified for the evaluation of HAAs and cancer. The protocol was informed by the evidence mapping and includes methods for evaluating study quality of the animal cancer studies as well as the approach for evaluating mechanistic data.

Cancer Hazard Evaluation

Evaluation of individual evidence streams. Data were first summarized for individual HAAs and evaluated for each type of evidence stream (e.g., properties, toxicokinetics, animal cancer studies, and mechanistic studies) (Figure 3, Step A). For the animal cancer studies, a structured systematic study quality evaluation was conducted, and conclusions were reached by applying the RoC listing criteria to the synthesis of the evidence across studies. Although a formal evaluation of the quality of mechanistic studies was not conducted, the mechanistic evidence was evaluated based on the overall strength of evidence with consideration of the depth of the available data, study design, and consistency of the results for each of the KCs.

Read-across, data trends, and grouping of chemicals. Next, the evaluation of the different evidence streams for individual HAAs was used to inform an assessment (read-across approach) to determine if any patterns of toxicity and carcinogenicity were related to structural classes of HAAs (see Figure 3, Step B, top). Several mechanistic studies that tested three or more HAAs concurrently in the same test system were particularly informative for the read-across approach because they helped identify data trends by directly comparing the toxicokinetics, chemical properties, and/or toxic effects across multiple HAAs (Larson and Bull 1992; Austin et al. 1996; Schultz et al. 1999; Kargalioglu et al. 2002; Walgren et al. 2004; Plewa et al. 2010; Zhang et al. 2010; Stalter et al. 2016; Zhang et al. 2016). Carcinogenicity potency estimates for the HAAs that induced cancer in experimental

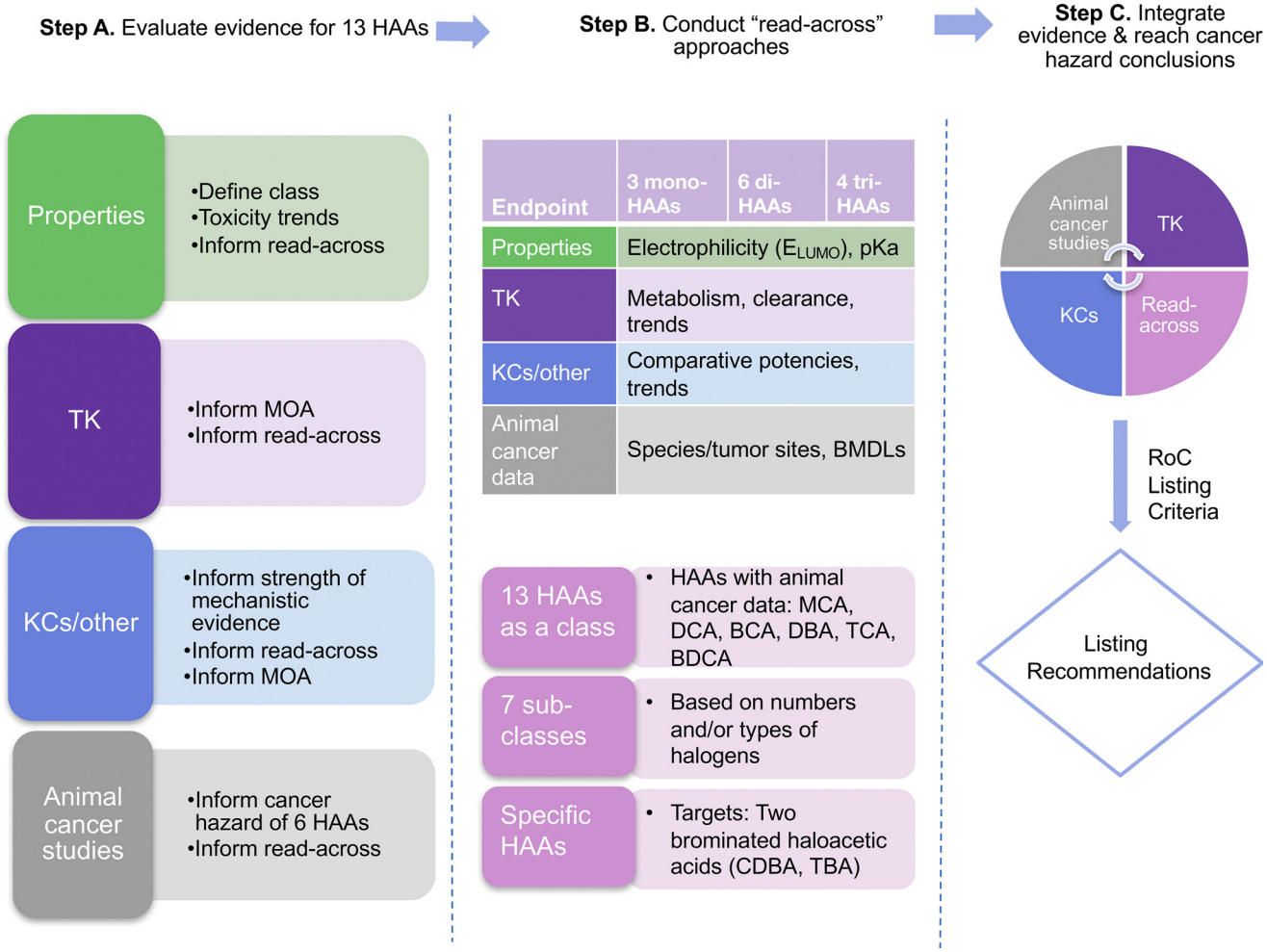

Figure 3. Cancer hazard evaluation approach for haloacetic acids (HAAs). The cancer hazard evaluation approach incorporated the following three steps: Step A: Evaluate evidence for 13 HAAs incorporating four primary evidence streams: *a*) properties, *b*) toxicokinetics, *c*) the key characteristics of carcinogens, and *d*) animal cancer studies. These data were then used to identify the properties that best correlated with toxic effects, inform the key events and potential modes of action, and determine the relative strength of the mechanistic evidence. Step B: Identify and implement read-across approaches. Step C: Integrate the evidence and propose listing recommendations. Note: BCA, bromochloroacetic acid; BDCA, bromodichloroacetic acid; BMDL, benchmark dose low; CDBA, chlorodibromoacetic acid; DBA, dibromoacetic acid; DCA, dichloroacetic acid; E_{LUMO}, energy of the lowest unoccupied molecular orbital; HAAs, haloacetic acids; KCs, key characteristics of carcinogens; MCA, monochloroacetic acid; MOA, modes of action; RoC, Report on Carcinogens; TBA, tribromoacetic acid; TCA, trichloroacetic acid; TK, toxicokinetics.

animals were available from U.S. Environmental Protection Agency (EPA)'s Integrated Risk Information System (IRIS) (U.S. EPA 2019a) and NTP's Chemical Effects in Biological Systems (NTP 2019a) database. Cancer potency estimates are expressed as the lower confidence limit of benchmark doses (BMDLs), which represent the 95% lower confidence limit corresponding to a 10% increased response level above controls. BMDLs were evaluated for correlations with chemical properties and KCs to determine if a QSAR model could predict cancer potencies of target HAAs.

NTP considered whether the read-across methods allowed *a*) grouping of all 13 HAAs as a class, *b*) grouping of one or more of 7 subclasses of HAAs based on the numbers and/or types of halogens (see section on "Potential class or subclass groupings" below), or *c*) identification of specific HAAs based on an analog approach (see Figure 3, Step B, bottom). The primary steps considered in the read-across evaluation included the following: *a*) source chemical identification, *b*) data identification and extraction for analogs, *c*) data evaluation, *d*) construction of a data matrix for source and target chemicals, *e*) assessment of the adequacy of the analogs to fill the data gaps, and *f*) documentation of the process (Patlewicz et al. 2013).

Evidence integration. NTP then used these evaluations to reach a listing recommendation for the RoC by applying the RoC criteria to the integration of the evidence for each of the data streams (animal cancer studies, toxicokinetic data, and KCs) and the read-across assessments (see Figure 3, Step C).

Peer Review

The draft RoC monograph and listing recommendations were presented to an expert panel of independent scientists on 24 July 2017 (NTP 2019b). This panel consisted of eight scientists (including a chair) with expertise in environmental health, pharmacology, physiology, pathology, and toxicology. Following the peer-review, the monograph was finalized based on the reviewer comments. The review process is shown in Figure 2.

Results

In this section, we discuss the key findings that were used to determine the recommendations for listing in the RoC; full details are reported in the final "Report on Carcinogens Monograph on Haloacetic Acids Found as Water Disinfection By-Products" (NTP 2018b). This includes brief reviews of the human and animal cancer data, mechanistic data, and read-across evaluation.

Human Cancer Data

Only one epidemiological study (Jones et al. 2017) was identified, and it reported no association between human kidney cancer risk and exposure to mixtures of HAAs, or to three individual HAAs (dichloro-, bromochloro-, and trichloroacetic acid) in disinfected water. Thus, the epidemiological data were considered inadequate to evaluate the relationship between exposure to HAAs and cancer in humans.

Animal Cancer Data

Four of the six HAAs with animal cancer data (dichloro-, dibromo-, bromochloro-, and bromodichloroacetic acid) were carcinogenic in rats and mice exposed via drinking water, and all four met RoC listing criteria for sufficient evidence of carcinogenicity (Herren-Freund et al. 1987; DeAngelo et al. 1996; Pereira 1996; DeAngelo et al. 1999; NTP 2007; DeAngelo et al. 2008; NTP 2009, 2015b; Wood et al. 2015; NTP 2018b). The three brominated HAAs with

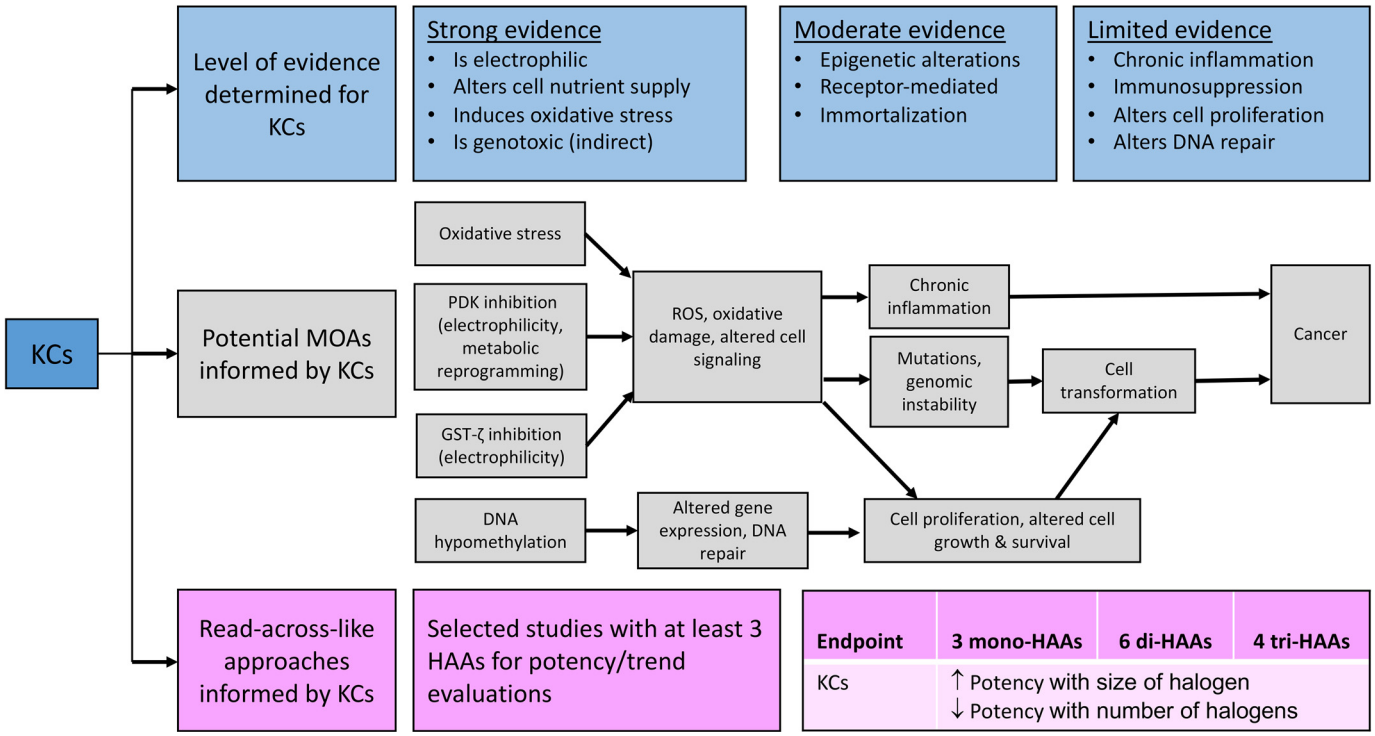

Figure 4. Results using key characteristics of carcinogens to inform mechanistic data evaluation and read-across. A systematic review of the literature identified several key characteristics of carcinogens (KCs) associated with the 13 haloacetic acids (HAAs). These data were used in three ways represented by the three rows in the figure: *a*) to determine the relative strength of evidence for each of the KCs (top row), *b*) to identify potential modes of action and key events associated with the KCs (middle row), and *c*) to identify studies that directly compared the potencies and trends for the KCs in three or more HAAs to inform read-across approaches (bottom row). Note: BCA, bromochloroacetic acid; BDCA, bromodichloroacetic acid; CDBA, chlorodibromoacetic acid; DBA, dibromoacetic acid; GST-ζ, glutathione S transferase zeta; HAAs, haloacetic acids; KCs, key characteristics of carcinogens; MOA, modes of action; PDK, pyruvate dehydrogenase kinase; ROS, reactive oxygen species; TBA, tribromoacetic acid.

animal cancer data were important for the read-across (discussed below). Chloroacetic acid was not carcinogenic in rats or mice (NTP 1992; DeAngelo et al. 1997), and trichloroacetic acid was carcinogenic only in mouse liver, which was insufficient to meet RoC listing criteria. Listings of individual studies reviewed and study quality assessments can be found in the final NTP monograph on HAAs (NTP 2018b).

Mechanistic Data

The mechanistic and health effects literature on HAAs is fairly extensive, although few data were available for the iodinated forms. Our evaluation revealed that the HAAs exhibited moderate to strong associations with most of the KCs (Figure 4), a characteristic that is shared with many IARC Group 1 and 2A carcinogens (Guyton et al. 2018). The KCs with the strongest experimental evidence, judged by available data for most or all of the HAAs and exhibiting consistent trends, included the following: *a*) they are electrophilic, *b*) they induce oxidative stress, *c*) they are genotoxic (although most likely an indirect effect), and *d*) they alter cell energy metabolism (i.e., nutrient supply). Other KCs with moderate support, judged by weakly positive to positive responses and fewer HAAs tested, included peroxisome proliferator–activated receptor alpha activation (a receptor-mediated effect examined for several HAAs but considered most relevant for trichloroacetic acid), epigenetic effects (DNA hypomethylation by dichloro-, dibromo-, and trichloroacetic acid), and cell transformation in NIH3T3 or Balb/c 3T3 cells (iodo- and dibromoacetic acid). In addition, toxicogenomic studies were also available for seven HAAs (chloro-, bromo-, iodo-, dichloro-, bromochloro-, trichloro-, and bromodichloroacetic acid) and indicated differential expression of genes that are directly relevant to several of the KCs listed above (e.g., oxidative stress and DNA damage response, cell proliferation, cell cycle control, apoptosis, cell metabolism, tumor progression, and metastasis) (Thai et al. 2001, 2003; Kim et al. 2006; NTP 2015a; Plewa and Wagner 2015; Lan et al. 2016). Although not specifically a KC, di-HAAs (primarily dichloroacetic acid) are known to suppress their own metabolism by glutathione S-transferase zeta, an effect directly related to electrophilicity.

The causal events leading to cancer are not fully known, and multiple initiating events are likely involved in the pathogenesis of cancer by HAAs, which complicates attempts to identify modes of action (MOAs) or adverse outcome pathways. Although the KCs do not necessarily represent mechanisms themselves or inform precisely what the specific events are and how they may be connected, the KCs can be used to identify, organize, and evaluate potential initiating and other key events that inform potential MOAs, as shown in Figure 4 (Smith et al. 2016). Evidential support for the proposed MOAs is strengthened in cases where temporal and dose-dependent progression can be demonstrated for the various KCs or other key events from the body of mechanistic evidence. HAAs cause cancer in several tissues and have a broad range of biochemical effects on those tissues. Cancer-initiating events likely involve electrophilic reactions with proteins and peptides rather than direct interactions with DNA (Stalter et al. 2016). These reactions lead to multiple key events that are associated with cancer, including inhibition of protein function, altered gene expression and DNA repair processes, altered cell growth and survival, oxidative stress, and metabolic reprogramming.

Read-Across Approaches

Data trends across haloacetic acids. HAAs are alkylating agents that react with cellular macromolecules (Plewa et al. 2004; Dad et al. 2018). However, the generally soft electrophilic character of HAAs suggests that the molecular initiating event is interaction with soft nucleophilic centers on proteins and peptides rather than a direct reaction with DNA (Stalter et al. 2016). Overall, the available studies consistently show that the toxicity of HAAs follows two general trends that are directly associated with their chemical reactivity and halogen substitution patterns: *a*) toxicity increases with increasing size of the halogen atom, i.e., $Cl \ll Br < I$; and *b*) toxicity decreases with the number of halogen substitutions, i.e., mono- > di- > tri- (Figure 4) (Richard and Hunter 1996; Plewa et al. 2010; Pals et al. 2011; Stalter et al. 2016). These trends were consistently reported for cytotoxicity, oxidative stress, genotoxicity, and developmental toxicity and were correlated with two chemical properties when they were considered together as independent variables (but not when considered alone): *a*) the energy of the lowest unoccupied molecular orbital (E_{LUMO}), a measure of electrophilicity; and *b*) the acid dissociation constant (pKa), a property related to chemical transport and bioavailability (Richard and Hunter 1996; Stalter et al. 2016).

Toxicokinetic studies of HAAs also revealed consistent patterns related to the number and types of halogens that were very important to the read-across evaluation. Although the rate of metabolism of HAAs shows interspecies differences (mice > rats > humans), the general trends are the same across species (Larson and Bull 1992; Tong et al. 1998; Gonzalez-Leon et al. 1999). In particular, the rate of metabolism and clearance increases as the number of bromines in the molecule increases, and all brominated tri-HAAs are metabolized to the di-HAA corresponding to the loss of a single bromine (Schultz et al. 1999; Saghir et al. 2011).

BMDLs were evaluated for correlations with chemical properties and KCs to determine if a QSAR model could possibly predict cancer potencies of target HAAs. However, the available BMDLs failed to identify clear potency trends because they were too similar, i.e., spanning only about one order of magnitude, and perhaps because they did not incorporate other factors such as the number of tumor types, systemic vs. local effects, species affected, time to tumor formation, or tumor multiplicity. However, it is clear that the brominated HAAs induced more tumor types than the chlorinated HAAs (dichloro- and trichloroacetic acid), and in that respect, the carcinogenicity data were consistent with the general toxicity trends (NTP 2018b; see Section 4). For example, compare dichloroacetic acid, which caused only liver tumors in rats and mice, with the multiple tumor sites in the brominated HAAs listed in Table 1.

Potential class or subclass groupings. Listing all 13 HAAs as a class was quickly ruled out because of data limitations and other uncertainties that included the following: *a*) chloroacetic acid (the only mono-HAA that had been tested for carcinogenicity in experimental animals) was negative, even though it did induce oxidative stress in mammalian cells and genotoxicity in bacteria and mammalian cells; *b*) existing data indicated that the toxicity of mono-HAAs is primarily due to disruption of energy metabolism by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition, while di- and tri-HAAs are weak GAPDH inhibitors (Dad et al. 2018); *c*) none of the iodinated HAAs have been tested for carcinogenicity, and data were limited overall for the di-HAAs that contained iodine; and *d*) uncertainty regarding the mode(s) of action.

Seven potential subclasses of HAAs (with overlap across several groups) were also evaluated, including *a*) mono-HAAs, *b*) di-HAAs, *c*) tri-HAAs, *d*) chlorinated acetic acids, *e*) brominated acetic acids, *f*) iodinated acetic acids, and *g*) brominated di- and tri-HAAs. The same uncertainties that prevented listing as a class were also relevant for the selected subclasses; thus, we ruled out listing any of these subclasses.

Read-across approaches for individual haloacetic acids. After eliminating the options to recommend listing HAAs as a class or subclass, we examined the properties, toxicokinetic, and KC data further to determine if an analog approach was possible to

Table 1. Tumor profiles in source chemicals and predicted tumor profiles in target chemicals.

	Source chemicals			Target chemicals	
Species/tumor type (sex)	BCA[a]	DBA[b]	BDCA	CDBA	TBA
Rat	+	+	+	Predicted	Predicted
Mononuclear cell leukemia (F)	–	+	–	–	–
Malignant mesothelioma (M)	+	+	+	(+)	(+)
Mammary (F)	+	–	+	–	–
Skin (M)	–	–	+	–	–
Mouse	+	+	+	Predicted	Predicted
Liver (M/F)	+	+	+	(+)	(+)
Lung (M)	–	+	–	–	–
Harderian gland (M)	–	–	+	–	–

Note: BCA, bromochloroacetic acid; BDCA, bromodichloroacetic acid; CDBA, chlorodibromoacetic acid; DBA, dibromoacetic acid; F, female; M, male; T, tri; +, tumor site; (+), predicted tumor site; –, not a tumor site.
[a]Metabolite of CDBA.
[b]Metabolite of TBA.

recommend listing any of the potential target HAAs (Patlewicz et al. 2015; Schultz et al. 2015). We identified two brominated HAAs (chlorodibromo- and tribromoacetic acid) as potential target chemicals based on their metabolism to source chemicals (bromochloro- and dibromoacetic acid, respectively) (Xu et al. 1995; Austin and Bull 1997; Saghir et al. 2011) and their similarity to bromodichloroacetic acid, an HAA that induced multiple tumor types in rats and mice (NTP 2018b). Overall, chemical similarities and trends in properties, toxicokinetics, and KCs provided a strong foundation for read-across from the source (tested) chemicals to the selected target (untested) chemicals (Table 1).

The toxicokinetic data indicated that substituting bromines for chlorine or adding bromines increased metabolism (Schultz et al. 1999), KC data indicated that substituting a bromine for a chlorine increased toxicity as determined by comparing potency values for various end points (Kargalioglu et al. 2002; Plewa et al. 2010; Stalter et al. 2016), and animal carcinogenicity data indicated that the carcinogenic response to brominated HAAs was enhanced compared with chlorinated analogs (NTP 2018b; see Section 4). All HAAs that contained at least two halogen atoms, at least one of which was a bromine, induced liver tumors in mice, malignant mesotheliomas in rats, and other extrahepatic tumors in mice and rats (see Table 1). In contrast, no extrahepatic tumors were induced by exposure to dichloro- or trichloroacetic acid (NTP 2018b). Thus, substitution of bromine for chlorine was an important determinant of the carcinogenicity of these compounds. This pattern is consistent with differences in toxicokinetics (e.g., increased metabolism with bromine substitution), increased reactivity and toxicity of bromine compared to chlorine, and the generally consistent trends reported for the HAAs and the KCs. We compare the overall data trends for the five brominated di- and tri-HAAs in Table 2.

Evidence Integration

Four HAAs (dichloro-, dibromo-, bromochloro-, and bromodichloroacetic acid) were recommended for listing as reasonably anticipated to be human carcinogens based on sufficient animal cancer data. NTP also recommended listing chlorodibromo- and tribromoacetic acid as reasonably anticipated to be human carcinogens in the RoC based on read-across from three source chemicals (Table 1).

Discussion

The NTP cancer hazard assessment of HAAs found as water disinfection by-products was one of the first monographs for which we used the 10 KCs to identify and organize mechanistic data and the first monograph to use read-across methods to evaluate chemicals that had not been tested for carcinogenicity in experimental animals. We believe that the lessons learned from this experience are applicable to other groups that conduct similar assessments. This section presents the strengths and limitations of the data and the overall approach, lessons learned and recommendations, and future directions.

Table 2. Comparison of properties and potency estimates for brominated di- and tri-haloacetic acids.

Parameters (units)	BCA	DBA	BDCA	CDBA	TBA
2-year cancer bioassay[a]	Yes	Yes	Yes	No	No
Total clearance (mL/kg/h)[b]	1,037	491	286	486	754
Renal	36.9	12.9	89	182	171
Non-renal	1,014	490	197	304	582
pKa[c]	1.4	1.39	0.05	0.04	0.03
E_{LUMO} (deprotonated)[d]	7.78	7.51	6.65	6.42	6.12
AREc32 ($1/EC_{IR1.5}$)[e]	7.1	8.3	0.5	0.2	2.3
ARE-bla ($1/EC_{IR1.5}$)[e]	2.2	4	0.25	0.46	1.5
8-OHdG (8OHdG/10^5 dG liver)[f]	2.9	2.9	1.7	—	—
TBARS (nmol MDA/g liver)[g]	290	250	240	—	—
Ames TA100 (-S9) (rev/μmol)[h]	—	183	—	—	0
Ames TA100 (-S9) (rev/μmol)[h]	60.6	61.9	31.6	1.7	1.2
Comet CHO cells (1/potency)[i]	333	556	0	71	400
P53-bla ($1/EC_{IR1.5}$)[j]	4,348	3,846	0	0	0
Tumor sites in rats or mice[k]	3	3	3	—	—
BMDL (1/mg/kg/d)[l]	0.08	0.04	0.06	—	—

Note: —, no data; BCA, bromochloroacetic acid; DBA, dibromoacetic acid; BDCA, bromodichloroacetic acid; CDBA, chlorodibromoacetic acid; TBA, tribromoacetic acid; TBARS, thiobarbituric acid-reactive substances.
[a]"Yes" for haloacetic acids tested in a 2-year cancer bioassay in experimental animals reported from NTP 2007, 2009, 2015b and "No" for those not tested.
[b]Data for total clearance, including renal and non-renal, from Schultz et al. 1999.
[c]Negative log of the acid dissociation constant from Stalter et al. 2016.
[d]Energy of the lowest unoccupied molecular orbital from Stalter et al. 2016.
[e]$1/EC_{IR1.5}$, reciprocal of the 50% effect increase in activation of the oxidative stress response pathway compared with the control, from Stalter et al. 2016.
[f]8 hydroxydeoxyguanosine from Larson and Bull 1992 and Austin et al. 1996;
[g]Thiobarbituric acid–reactive substances, and MDA, malondialdehyde, from Larson and Bull 1992 and Austin et al. 1996.
[h]Rev/μmol, revertants per micromole (adjusted for cytotoxicity) for Ames assay, from Kargalioglu et al. 2002 and Plewa et al. 2004 for upper row and NTP 2019a for lower row.
[i]Chinese hamster ovary cells, 1/potency, i.e., reciprocal of potency, from Plewa et al. 2010.
[j]$1/EC_{IR1.5}$, reciprocal of the 50% effect increase in activation of the tumor suppressor protein p53 compared with the control, from Plewa et al. 2010.
[k]Multiple tumor sites in rats or mice.
[l]Benchmark dose low; reported as 1/BMDL, i.e., reciprocal of BMDL, from U.S. EPA 2019a and NTP 2019a.

Strengths and Limitations of Available Data

The HAAs offered an excellent test case for the NTP to use KCs as a practical framework for identifying, screening, organizing, and evaluating mechanistic data and to apply read-across principles. HAAs are simple, two-carbon molecules that differ only in the halogen substitution patterns. The chemistry of the halogens affected the toxicokinetics, reactivity, and biological effects in a consistent fashion. Toxicokinetic and mechanistic data were available for all 13 HAAs reviewed and were critical to adopting a read-across approach. Dichloroacetic acid was by far the most extensively studied, while limited information was available for most of the iodinated HAAs. Several potential key events were identified that suggested possible cancer pathways, especially when combined with generally consistent trends in metabolism and biological effects. These trends, in turn, along with the positive animal carcinogenicity data for 5 of the 13 HAAs, were critical for supporting read-across in the overall hazard assessment and listing recommendations. As a result, two HAAs without cancer studies were proposed for listing in the RoC.

Overall, these data indicated that the mechanisms by which HAAs induce cancer in experimental animals are variable and highly complex, likely involving multiple interactions among the various KCs, toxicokinetics, and other factors. For example, some of the HAAs (dichloro- and trichloroacetic acid) did not appear to share the same cancer pathways based on species affected and tumor pathogenesis. The lack of a known cancer mode of action for all 13 HAAs considered in the RoC monograph was a major factor in ruling out a recommendation to list as a class or subclass and was a primary source of uncertainty in the overall hazard assessment.

Other limitations of the available data on HAAs for read-across were the reliance on *in vitro* studies, the limited number of *in vivo* genetic toxicity or other mechanistic data available, the lack of a clear trend in the BMDLs as a measure of cancer potency, and a negative cancer bioassay for monochloroacetic acid (the only mono-HAA tested for carcinogenicity). In addition, aside from physical–chemical properties and a positive cell transformation assay for monoiodoacetic acid, few cancer or mechanistic data were identified for the iodinated HAAs. These limitations were the primary factors that prevented further consideration of the other mono-HAAs or di- and tri-iodinated HAAs as carcinogens and also prevented using a category read-across approach to recommend listing HAAs as a class or any subset of HAAs as a subclass.

Strengths and Limitations of Approach

We believe that focusing the literature search on the KCs contributed to a more thorough review of the available mechanistic evidence and likely avoided potential biases that might have been introduced if the assessment had focused on specific mechanistic hypotheses. A primary strength of this process was the added value when expanded to inform the potential cancer pathways and read-across approaches (Figure 4). Overall, a systematic review based on the KCs offered a feasible and transparent approach that was successfully applied to evaluate mechanistic evidence of carcinogenicity.

A number of well-known challenges are associated with identifying and evaluating mechanistic data in chemical hazard assessments. These challenges include identifying all relevant studies, efficiently extracting and screening data from potentially numerous and heterogenous studies, and assessing the internal and external validity of existing and emerging *in vitro* and *in silico* studies. Therefore, the strategies and tools for conducting systematic reviews are not as well developed as they are for disease or adverse effects studies in humans and animals. Although we have implemented a full systematic review approach for human and animal cancer data (i.e., literature search and study selection, data extraction, study quality and utility, and level of evidence integration), our systematic review methods for mechanistic data were limited to literature search, study selection, and professional judgment. We are currently developing or adapting existing tools to extract data and assess the internal validity of mechanistic studies in a more formal and transparent manner.

Lessons Learned and Recommendations

The primary lesson learned in our cancer hazard assessment for HAAs is that the KC approach was effective, and we recommend this approach for any agency or group charged with conducting cancer hazard assessments when appropriate data are available for a set of chemicals. As noted above, a critical factor in our decision that HAAs could be a suitable candidate to apply read-across methods to cancer hazard assessments was the use of a systematic approach to the literature searches and evidence mapping based on the 10 KCs and toxicokinetics. It is also important to evaluate any available information on toxicokinetics for the chemicals of interest since metabolism of the two target HAAs was an important factor in the recommendation for listing in the RoC. Evidence maps suggested that there was sufficient mechanistic and metabolic information to extrapolate from chemicals that had cancer data to those without that information. The evidence maps and subsequent study reviews identified the strengths and weaknesses of the mechanistic evidence, the potential importance of the biological effects or events associated with the KCs and their relationship to the halogen substitution patterns, and data gaps. Collectively, this information provided the overall structure that guided selection of potential tools (i.e., read-across approaches and/or QSAR) and identified potential MOAs. In our experience, heat maps of extracted data further defined potential chemical subgroup patterns, and by constructing a heat map of chemicals vs. properties and potency values for biological effects, general relationships and trends among the chemical groups were more apparent. The heat map also served to identify the chemicals that were data sparse and could be used to direct future research. In addition, we were fortunate to have animal cancer data for 6 of the 13 HAAs and some toxicokinetic and mechanistic data for all 13 HAAs. The data included comparative studies of multiple chemicals within a multiplex assay system from several laboratories that showed consistent results, thus reducing concern for bias or differences between assays and/or laboratories.

Future Directions

Cancer hazard assessments have long relied on human epidemiology studies and the lifetime rodent bioassay; however, it is clear to all stakeholders that more cost- and time-efficient methods are needed to keep pace with the growing demands for such assessments while protecting public health. In an effort to meet this challenge, health research organizations and regulatory agencies around the world are actively involved in the development and validation of alternative methods and tools to better utilize mechanistic data in hazard and risk assessments. We are currently evaluating approaches and tools under development by regulatory agencies and other hazard assessment groups for efficiently extracting and evaluating the quality of mechanistic data, and we will be updating our approach to the systematic review of mechanistic data for hazard evaluation based on these reviews and experiences. In addition, we are exploring the application of read-across tools for future assessments and also the use of computational modeling methods, such as physiologically based toxicokinetics to evaluate associations between *in vitro* bioactivity and *in vivo* toxicity (Honda et al. 2019). A potential QSAR was evaluated in the current HAA assessment, but the available data were considered inadequate for useful predictions. Nevertheless,

this is a promising tool for read-across, and future efforts will include this option.

In this commentary, we report on a recent case study where mechanistic data, organized by KCs, supported a limited use of read-across in an NTP cancer hazard assessment. Our efforts to incorporate systematic review, KCs, and read-across approaches to hazard assessment are consistent with developments in other programs within the NTP, EPA, IARC, and other hazard assessment groups. These agencies and others are actively involved in developing, validating, testing, and improving methods and tools to support increased use of mechanistic data in chemical hazard and risk assessments. Expanded availability and use of high-quality mechanistic data, along with standardized frameworks and guidelines, will facilitate consistent and transparent application of data evaluations, expedite and improve chemical hazard assessments, and potentially reduce reliance on animal testing.

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